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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Beat MOLLET et al.

Application No.: 09/823,772

Filed: March 30, 2001

For: NOVEL STRAINS OF THE BACILLUS SUBTILIS GROUP FOR FOOD FERMENTATION

Confirmation No. 6428

Group Art Unit: 1652

Examiner: R. Hutson

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SUBMISSION OF CERTIFIED PRIORITY DOCUMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants have claimed priority of European application no. EP 98118659.6 filed October 2, 1998, under 35 U.S.C. § 119. In support of this claim, a certified copy of said application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn Deposit Account No. 501-814.

Respectfully submitted,

Date: 3/24/03

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Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

98118659.6

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

DEN HAAG, DEN
THE HAGUE, 12/03/03
LA HAYE, LE

Eur päisches Patentamt
Eur pean Patent Office
Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Demande n°: 98118659.6

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Anmelder:
Applicant(s):
Demandeur(s):
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Novel strains of the bacillus subtilis group for food fermentation

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Novel strains of the Bacillus subtilis group for food fermentation

The present invention pertains to novel strains of Bacillus subtilis capable of fermenting beans, which are essentially devoid of any iso-valeric acid production. The present invention especially relates to novel strains of Bacillus natto, in which one or more genes involved in the biosynthetic pathway for the production of iso-valeric acids are essentially non-functional.

For the production of food material mankind has long since used various micro-organisms, such as yeasts, fungi or bacteria, so as to modify, prepare or change the nature/taste of foodstuff. One such kind of microorganisms are the soil bacteria belonging to the Bacillus subtilis group that are used for the fermentation of different plants tissues, such as beans, e.g. soybeans and (African) locust beans, and seeds, e.g. the seeds of African oil bean, cotton seeds, melon seeds etc.. In the fermentation process B. subtilis degrades cellulose or/and the protein material contained in the starting material resulting in a fermented product that may be further processed or is already ready for consumption.

One bacteria very closely related to B. subtilis is the so called strain B. natto, a food-grade, gram-positive microorganism mainly used for the fermentation of soybeans, which fermentation process eventually results in a cheap and nutritious food that is rich in amino acids. The term B. natto is derived from the Japanese soybean fermented product "Natto" that is commercially produced and often eaten at breakfast (see also K.H. Steinkraus et al., Handbook of Indigenous Fermented Food, Vol. 9. (1983), 530-547).

A drawback in the fermentation of biological starting materials with micro-organisms for food production resides in that a variety of by-products are generated that are not desirable by the consumer, such as an off-flavor or an unwanted hardness of the product.

To this end the JP-08275772 describes the use of a particular strain of B. subtilis for reducing the amount of ammonia in the end product "Natto". This objective is achieved by keeping the protease activity during the early stage of fermentation at

a high level, so that essentially all soybean proteins are degraded to a substantial degree, while in a later stage of fermentation the protease activity is remarkably reduced so as not to produce extensive amounts of ammonia which would eventually deteriorate the smell of the food product.

In JP-09009903 there is described another B. subtilis strain that has improved hemicellulose degrading properties such that the end product reveals an increased softness.

However, though the properties of food products derived from a fermentation with microorganisms, such as B. subtilis, have been improved in various respects there is still a need for a further improvement of the taste and/or smell of the final end product.

The object of the present invention therefore resides in further improving the properties of food products obtainable by a fermentation with B. subtilis, especially improving the taste thereof.

The above object has been solved by providing a novel strain of a microorganism of the B. subtilis group capable of fermenting beans, preferably soybeans, which does not produce substantial amounts of iso-valeric acid.

In the Figures:

Fig. 1 shows a flowchart for the construction of an *ywfL* dispruption product.

Fig. 2 shows the recombinant vector pMZ66 harbouring the *ywfL* dispruption product of fig. 1.

Fig. 3 shows a chromatogram of fermentation products produced by the wild type Bacillus natto and the *ywfL* dispruption isogenic derivative.

In the extensive experiments leading to the present invention it has been found that the flavour of products obtainable by fermentation with B. subtilis, especially B. natto, are unfavourably affected by particular compounds produced by the microorganism during its propagation, namely the iso-valeric acids (2-methyl-

butyric acid and 3-methyl-butyric acid), which compounds result in a clinging, strong and pungent smell of the fermentation product.

It is known that in microorganisms the major use of branched chain fatty acids, such as the iso-valeric acids, resides in the synthesis of the cell membrane where they account for approximately 90 % by composition. Cell membrane synthesis is an essential function of any cell. Hence, influencing the biosynthesis of one of its components was expected to be a delicate matter, since a decreased production of any of the fatty acids required for the formation of the cell membrane or even an entire depletion thereof may eventually lead to microorganisms not viable under normal conditions or not able to fulfill their function as a fermenting agent.

Although the exact biosynthesis pathway for iso-valeric acids by B. subtilis is not known a potential synthesis pathway has been devised considering the information from the newly completed total genome of B. subtilis (Kunst, F. et al. "The complete genome sequence of the gram positive bacterium B. subtilis", Nature **390** (1997), 249-256).

To this end, it was also assumed that the polypeptide derived from the gene *ywfL* may play a vital role in the production of iso-valeric acids and similar branched-chain fatty acids by the bacterium.

In order to achieve the above object, in the subject B. subtilis strains one ore more genes involved in the biosynthesis of iso-valeric acids are rendered essentially non-functional, so that the respective gene products thereof do show a comparatively reduced activity or may essentially not be translated to the gene products at all.

In a preferred embodiment this may e.g. be achieved by modifying one or more of the genes involved in the biosynthetic pathway for the synthesis of iso-valeric acids, preferably the *ywfL* gene (Nature, supra) in such a way that the gene product(s) thereof reveal merely a reduced activity, preferably a strongly reduced activity or are non-functional. These gene products may comprise polypeptides acting as enzymes within the synthesis pathway or acting as regulatory agents for the production of isovaleric acids. In a preferred embodiment the *ywfL* gene may

be deleted from the genome or is modified such that the gene is not transcribed into a functional protein.

In a further preferred embodiment the modified strain belonging to the B. subtilis group is of the species B. natto, most preferably B. natto BN10, that has been deposited with the Institute Pasteur under the Budapest Treaty having received the deposit number I-2077.

With respect to the objective to use the novel B. subtilis strains in foodstuff it is further preferred that no exogeneous sequences, such as vector sequences or genes coding for selection markers, as e.g. antibiotic resistances, are contained in the B. subtilis strains. This applies likewise to the presence of such sequences as extra-chromosomal DNA or DNA integrated into the chromosome.

The novel strains may be obtained by known techniques, such as mutating common B. subtilis strains with known mutagens and selecting for the desired trait, that is a low or deficient synthesis of iso-valeric acids during fermentation. Mutagens and techniques for applying them are well known in the art and not limiting examples are e.g. DMSO (dimethylsulfoxide), MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), methylamine or radiation treatment etc..

Moreover, the objective B. subtilis strains may also be obtained by recominant gene technology, preferably without any exogeneous DNA incorporated therein, which will be described in detail hereunder.

The novel B. subtilis strains according to the invention may be used for the fermentation of plant material to eventually produce therefrom foodstuff, flavours or, more preferably, Natto.

In the following the construction of a novel and stable, food-grade, genetically modified organism, B. natto, is described that contains an isogenic deletion of the chromosomal *ywfL* gene. This deletion has been found to be stable and contains no undesired DNA sequences, such as vector sequences or antibiotic resistance markers used for its construction. Moreover, the microorganisms obtained by deleting the *ywfL* gene have shown to perform equally well as compared to known

B. natto strains indicating, that the fermentation behaviour of the novel strains are not deteriorated by the lack of the *ywfL* gene product.

Unless otherwise indicated all techniques, conditions and media are as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1992), Cold Spring Harbor Laboratory press, NY).

Plasmids, bacterial strains and media

DNA amplification of the (recombinant) E. coli vector pBS SK+ (Stratagene, product number 212205) was carried out in E. coli strain BZ234 (Mollet, B. et al. "Directed genomic integration, gene replacement, and intgrative gene expression in Streptococcus thermophilus" Journal of Bacteriology **175**(14) (1993), 4315-4324) while selecting for recombinant strains by means of the antibiotic ampicillin (Boehringer Mannheim, product number 835 242). Recombinant plasmids were identified with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer Mannheim, product number 1 680 293) and IPTG (isopropyl-β-D-thiogalactoside, Boehringer Mannheim, product number 1 411 446).

The plasmid pG+host9 (Maguin, E. et al. "Efficient insertional mutagenesis in lactococci and other gram-positive bacteria" Journal of Bacteriology **178**(3) (1996), 931-935) is a gram-positive / gram-negative shuttle vector harbouring a gene for the resistance to the antibiotic erythromycin (Fluka, product number E6376) and a temperature sensitive plasmid replication protein. pG+host9 is propagated in E. coli EC101 (Law, J. et al. "A system to generate chromosomal mutations in Lactococcus lactis which allows fast analysis of targeted genes" Journal of Bacteriology **177**(24) (1995), 7011-7018) which provides the non-temperature sensitive replication protein integrated in the genome for convenient maintenance and amplification of the plasmid.

The B. natto strain (termed BN1) used in this work has been isolated from a fermented Natto product purchased on the market.

The growth medium was LB at 37°C for E. coli and either 28°C or 37°C for B. natto with agitation. Erythromycin was added to 150 µg/ml for E. coli and 2-4 µg/ml for B. natto.

B. natto chromosomal DNA extraction

The extraction of chromosomal DNA from B. natto for PCR and Southern blot analysis was performed on a 16 hr culture in LB medium with or without antibiotic selection as required. The culture was centrifuged at 6,000 rpm for 8 min to pellet the bacteria. The pellet was suspended in 500 µl of 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA plus 500 µg/ml lysozyme (Boehringer Mannheim, 1243004) and incubated at 37°C for 30 min. Mutanolysin (Fluka, M9901) was added to 1 µg/ml and the incubation was continued at 37°C for another 30 min. Proteinase K was added (Fluka, P6556) to 20 µg/ml, EDTA to 2.5 mM and the cells were finally lysed by the addition of 0.1 % SDS (Serva, 20763). This solution was incubated at 60°C for 1 hr and the lysate was extracted once with an equal volume of phenol-chloroform. The mixture was centrifuged at 14,000 rpm for 8 min to separate the phases. The aqueous phase was carefully removed and the chromosomal DNA was precipitated by the addition of 2 volumes of 95% ethanol (Fluka, 02860). The DNA precipitate was spooled with a sterile toothpick, transferred to 400 µl 10 mM Tris-HCl pH 8.0, 10 mM EDTA with 50 µg/ml RNAse (Boehringer, 109 169) and incubated at 60°C for 1 hr. The solution was phenol-chloroform extracted. The DNA was precipitated, spooled and finally suspended in 200 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Construction of the *ywfL* deletion plasmid pMZ66

DNA fragments flanking the *ywfL* gene were amplified from the B. natto strain BN1 and finally combined (Fusion Cycle PCR) by their primer incorporated homologies to create a DNA fragment for the disruption of the *ywfL* gene in this bacterium. This is schematically shown in Figure 1.

The 5' flanking region was amplified using the oligonucleotides 6624 (SEQ. ID. NO.1) obtained from Microsynth, Balgach, Switzerland, which introduces a BamHI restriction site approximately 950 bp upstream of the start of the *ywfL* gene, and 6626 (SEQ. ID. NO. 2), a composite oligonucleotide comprising 22 bp of the sequence of the region 50 bp away from the start of the *ywfL* gene and 22 bp of the region 100 bp away from the end of the *ywfL* gene. This oligonucleotide

sequence defines the region that is to be deleted, namely the sequence that is omitted between the two segments of the composite primers. This second segment from the oligonucleotide 6626 was also designed to introduce two TGA stop codons to terminate translation of the truncated *ywfL* gene.

The 3' flanking region was amplified using the oligonucleotides 6625 (SEQ. ID. NO. 3), which introduces a EcoRI restriction site approximately 1000 bp downstream of the end of the *ywfL* gene, and 6627 (SEQ ID. NO. 4) a second composite oligonucleotide that is essentially the reverse-complement of oligonucleotide 6626 (SEQ. ID. NO. 2). These complementary oligonucleotide sequences provide the homology between the the 5' and 3' fragments that is exploited in the primer-free, Pwo directed extension to complete the second strand. The two oligonucleotides 6626 and 6625 were finaly added and the correct deletion fragment was specifically amplified.

The PCR reactions were carried out as follows. 500 ng of chromosomal DNA were mixed in a 100 µl volume containing 80 µl sterile H_2O, 6 µl 2 mM dNTPs, 10 µl Pwo polymerase reaction buffer, 2 µl each oligonucleotide at approximately 100 nM and 0.5 µl Pwo polymerase (Boehringer Mannheim, product number 1 644 947). The desired fragment was amplified using a Perkin Elmer DNA Thermal Cycler with 20 cycles of 1 min 95°C, 1 min 40°C, 2 min at 72°C and finally held at 4°C. A 1 µl sample from each PCR reaction was prepared in a 100 µl volume containing 80 µl sterile H_2O, 6 µl 2 mM dNTPs, 10 µl Pwo polymerase reaction buffer and 0.5 µl Pwo polymerase (without any oligonucleotides). This control was processed for two cycles in the Thermal Cycler as described above to extend from the oligonucleotide induced homologies. Finally, 2 µl of each oligonucleotide 6624 and 6625 at approximately 100 nM were added and the PCR reaction was continued for another 20 cycles.

The final PCR product was purified on a QIAquick PCR purification kit (Qiagen, product number 28104). A sample was digested with the restriction enzymes EcoRI and BamHI and electrophoresed on a 1% agarose gel. The corresponding 2 kb fragment was cut out of the gel and the DNA was eluted using the QIAquick gel extraction kit (Qiagen, product number 28704). The DNA fragment obtained was digested with the restriction enzymes EcoRI and BamHI and ligated with the E. coli vector pBS SK+ that has been pretreated accordingly (digested with

EcoRI/BamHI and dephosphorylated). The ligation mixture was electro-transformed in the E. coli strain BZ234 and transformants were selected on LB plates supplemented with 100 µg/ml ampicillin, Xgal and IPTG. Potential positive, white colonies were screened by restriction analysis of plasmids isolated therefrom (Sambrook, supra). The DNA sequence of the insert of a positive clone was determined so as to confirm the PCR construction. This plasmid was digested with the restriction enzymes EcoRI and SpeI and the fragments were separated on a 1% agarose gel. The corresponding 2 kb fragment was cut out of the gel and the DNA was eluted using the QIAquick gel extraction kit.

The replication-temperature sensitive vector pG+Host 9 was digested with the restriction enzymes EcoRI and SpeI and the terminal phosphates were removed using shrimp alkaline phosphatase (USB, product number 70092). The *ywfL* deletion fragment was mixed with the pG+Host 9 vector pretreated accordingly and ligated therwith. The ligation mixture was electro-transformed into the E. coli host EC101. Colonies were screened by means of restriction analysis of isolated plasmids. One of the positive plasmids was designated pMZ66 (Figure 2).

For the transformation into BN1, a large quantity of pMZ66 was isolated using the Jetstar Maxi prep kit (Genomed, 220010).

Transformation of B. natto

The transformation experiments were carried out according to the protocol:

Solutions:
Medium I: Spizizen's Salts 5x, 2 ml; glucose 50%, 0.1 ml; casamino acids 20%, 0.01 ml; yeast extract 5%, 0.02 ml; $MgSO_4$ 1 M, 0.05 ml; adjusted to 10 ml with distilled H_2O.
Medium II: Spizizen's Salts 5x, 2 ml; glucose 50%, 0.1 ml; casamino acids 20%, 0.005 ml; $MgSO_4$ 1M, 0.05 ml, adjusted to 10 ml with distilled H_2O.
Spizizen's Salts 5x: $(NH_4)_2SO_4$ 10 g, K_2HPO_4 70 g, KH_2PO_4 30g, Na_3-citrate.$2H_2O$ 5g, $MgSO_4$.7 H_2O 1 g, filled up with distilled H_2O to 1 liter.

Natural competency of B. natto

2-3 colonies from LB plates incubated overnight at 37°C were re-suspended into 2.5 ml of Medium I and incubated at 37°C with aeration (240 rpm) in a sterile 10 ml glass tube for 4 to 5 hours.

Transformation of B. natto

A ten times dilution was made in Medium II (0.05 ml in 0.45 ml) with the addition of plasmid DNA (5 to 10 μg in maximum 50 μl). Incubation was made at 30°C, overnight, with aeration (240 rpm). Aliquots or the whole volume were then plated onto selective medium (LB with 4 μg/ml Erythromycin for pG+host9) and were incubated at 28°C for two days.

The deletion of the B. natto *ywfL* gene was performed in two separate steps. In the first step (Loop-in) the integration of pMZ66 by homologous recombination (directed by the flanking DNA homologies) was selected for. In the second step (Loop-out) use was made of plasmid replication facilitated excision from the genome and the desired bacterial clones were identified.

Loop-in of pMZ66.

The B. natto strain BN1 transformed with the plasmid pMZ66 was inoculated into fresh LB medium supplemented with 2 μg/ml erythromycin and was incubated at 42°C for 16 hr. This culture was diluted and plated onto on LB plates supplemented with 2 μg/ml erythromycin and incubated at 42°C. At this temperature the pG+Host 9 plasmid replication protein is no longer active. Consequently, those bacteria, that are selected, are the rare events of plasmid integration at the *ywfL* gene. This integration is directed by the DNA sequence homology of the *ywfL* deletion fragment and can occur in either the 5' or 3' homology region. The event of either the 5' or the 3' integration was determined using specifically designed PCR primers on small-scale cultures of the integrants (at 42°C). This revealed that the majority of the chromosomal integration events occurred at the 5' section of the *ywfL* gene, with only approximately 10 % of events occurring at the 3' end. These clones were confirmed by Southern analysis.

Loop-out of pMZ66.

A positive clone with pMZ66 integrated at the 5' end of the *ywfL* gene was inoculated at 1% into LB medium with 2 μg/ml erythromycin and was incubated at 42°C for 16 hr. This culture was then used to inoculate at 1 % a fresh culture of LB medium and incubated at 28 °C for 16 hr. The culture was diluted, plated onto LB plates and then incubated at 42°C.

The reasoning for proceeding accordingly was as follows:
At 28°C the pG+Host 9 plasmid replication protein is again active and the re-establishment of replication enhances the excision of the plasmid from the genome, while the final plating and the incubation at 42°C again shuts-off the pG+Host 9 plasmid replication protein causing the freely replicating plasmid to be lost (no erythromycin selection).

As in the loop-in reaction, the plasmid pMZ66 is considered to have two options for looping-out by two distinct routes:
i) by recombination within the same 5' flanking DNA, as with the integration, thus returning to the original parent BN1, or
ii) by recombination within the 3' flanking DNA, that is by incorporating the deletion fragment into the genome and removing the chromosomal *ywfL* gene with the pG+Host 9 vector.

The resulting colonies incubated at 42°C showed predominantly large colonies with a few smaller colonies being present. Replica streaking onto LB plates and LB plates supplemented with 2 μg/ml erythromycin determined that all of the small colonies and some of the large colonies tested were erythromycin sensitive.

PCR amplification with primers designed to amplify across the deletion point were used to determine that all the large colonies carried the wild-type BN1 *ywfL* gene, while the small colonies all contained the designed deletion of the *ywfL* gene.

The PCR results were confirmed by sequencing the DNA at the deletion point of the *ywfL* gene, which showed the expected sequence from the constructs. The arrangement of the region around the *ywfL* gene was confirmed by Southern hybridization and finally it was determined by means of hybridization with that

plasmid that no pG+Host 9 vector sequences remained. Five such *ywfL* deletion strains were identified from independent experiments and named BN10 (I-2077) to BN 14.

HPLC analysis of *ywfL* deletions strains.

B. natto strain BN1 and the 5 *ywfL* deletion strains obtained (BN10 to BN14) were cultured in LB medium for 16 hr at 37°C. The bacteria were removed by filtration through a 0.2 micron filter (Schleicher & Schuell, FP 030/3) and the fatty acids composition was analysed by HPLC. HPLC was performed on a HPLC Hewlett Packard series 1100 machine using an Aminex Fast Acid 100 x 7.5 mm column (Bio-Rad, product number 125-0100) stabilized at 40°C and eluted with 10 mM H_2SO_4 at a flow rate of 1 ml/min. Detection was achieved using a HP1047A Refractometer (Hewlett Packard) also stabilised at 40°C.

The results are presented in Table 1, from which it becomes evident that the iso-valeric acids is produced by the fermentation of the bacterium B. natto, and the deletion of the *ywfL* gene extensively reduces the production of this fermentation product to a minimum.

Table 1

Determination of iso-valeric acid levels produced by the BN1 and 5 *ywfL* deletion derivatives BN10-BN14

Sample	iso-valeric acid (mg/l) (3-methyl-butyric acid)	iso-valeric acid mmoles/l
LB medium	0	0
BN1	971.7	9.51
BN10	< 7.0	< 0.07
BN11	< 7.0	< 0.07
BN12	< 7.0	< 0.07
BN13	< 7.0	< 0.07
BN14	< 7.0	< 0.07

The following examples illustrate the invention and are not considered to limit the scope of the appended claims.

Example 1

Preparation of cubes

According to techniques well known in the art soybeans were crushed, cooked, and inoculated with spores of the B. natto strain BN10 (I-2077), followed by solid state fermentation (Koji type fermentation) for 2 - 5 days at 30-50°C. To the resulting fermentation mixture was added a salt brine (NaCl saturated solution). The product was dried, pressed into cubes or was used as the powder for e.g. bouillon production. The final production did not have the taste common for products containing iso-valeric acids.

The novel B. subtilis strain therefore performed equally well in the fermentation of soybeans as compared to commonly used B. subtilis strains.

Example 2

The same procedure as illustrated in example 1 was repeated with the proviso of using a wild type B. natto (BN1) for inoculation. The resulting product showed a taste typically for products containing iso-valeric acids.

Claims

1. A bacterial strain of the B. subtilis group capable of fermenting beans, which does not produce substantial amounts of iso-valeric acids.

2. The B. subtilis strain according to claim 1, which is B. natto.

3. The B. subtilis strain according to claim 1 or 2, wherein one or more gene products derived from genes involved in the biosynthesis of iso-valeric acids have a reduced activity, are essentially non-functional or are missing.

4. The B. subtilis strain according to claim 3, wherein the gene product that has a reduced activity, is essentially non-functional or is missing is derived from the *ywfL* gene.

5. The B. subtilis strain according to any of the preceeding claims, that contains no exogeneous DNA sequences.

6. The B. subtilis strain according to any of the preceeding claims, that has been prepared by recombinant gene technology.

7. The B. subtilis according to any of the claims 1 to 5, that has been prepared by mutagenesis and selection.

8. The B. subtilis strain according to claim 6 which is B. natto I-2077.

9. Use of a B. subtilis according to any of the preceeding claims for the fermentation of plant material.

10. The use according to claim 9 for the preparation of foodstuff.

11. The use according to claim 9 for the preparation of flavour compounds.

12. The use according to claim 10, for the preparation of Natto.

Figure 1. Schematic of the PCR reactions to construct the DNA fragment for the deletion of the *ywfL* gene in *B. natto* strain BN1.

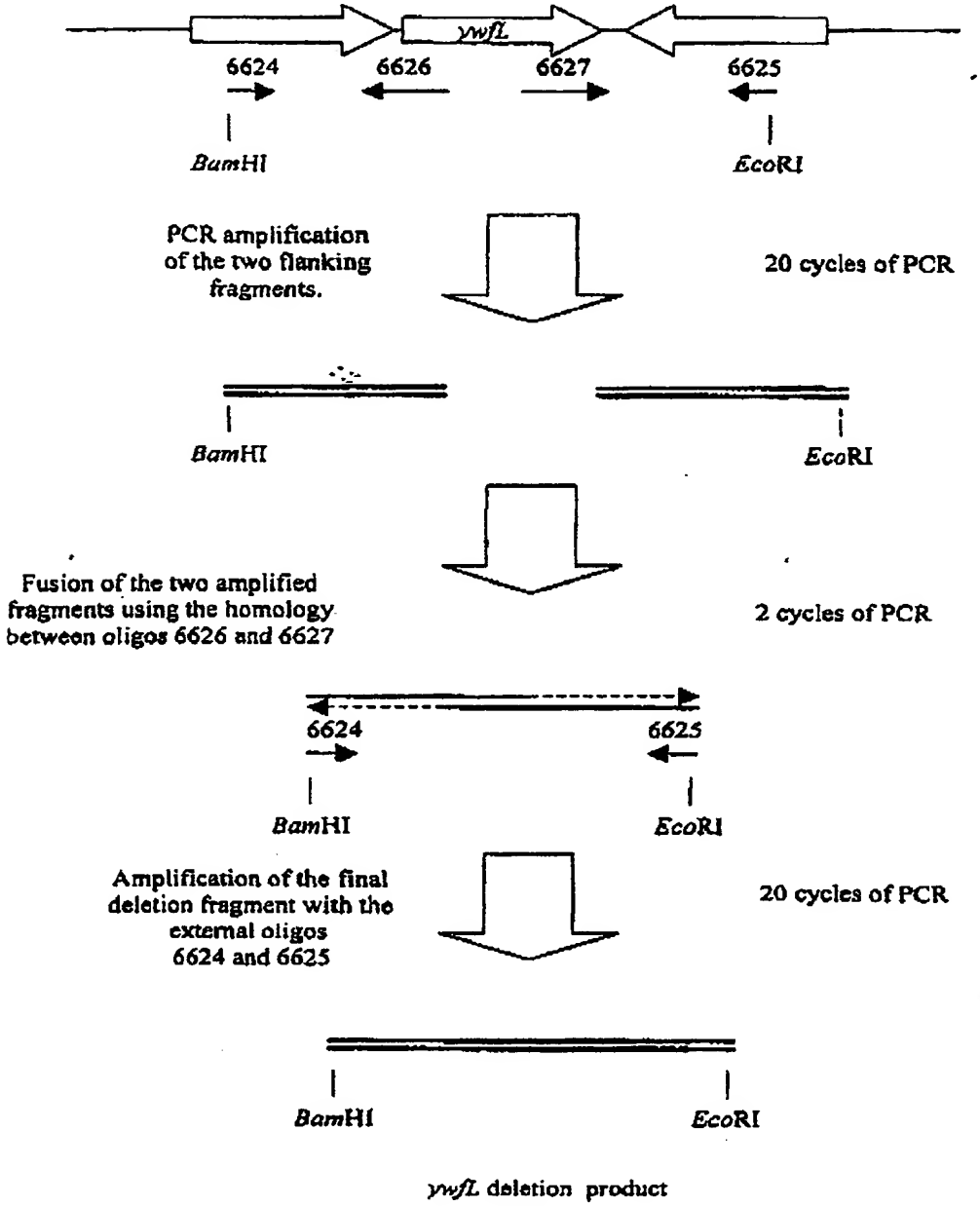


Figure 2. Schematic map of the plasmid pMZ66.

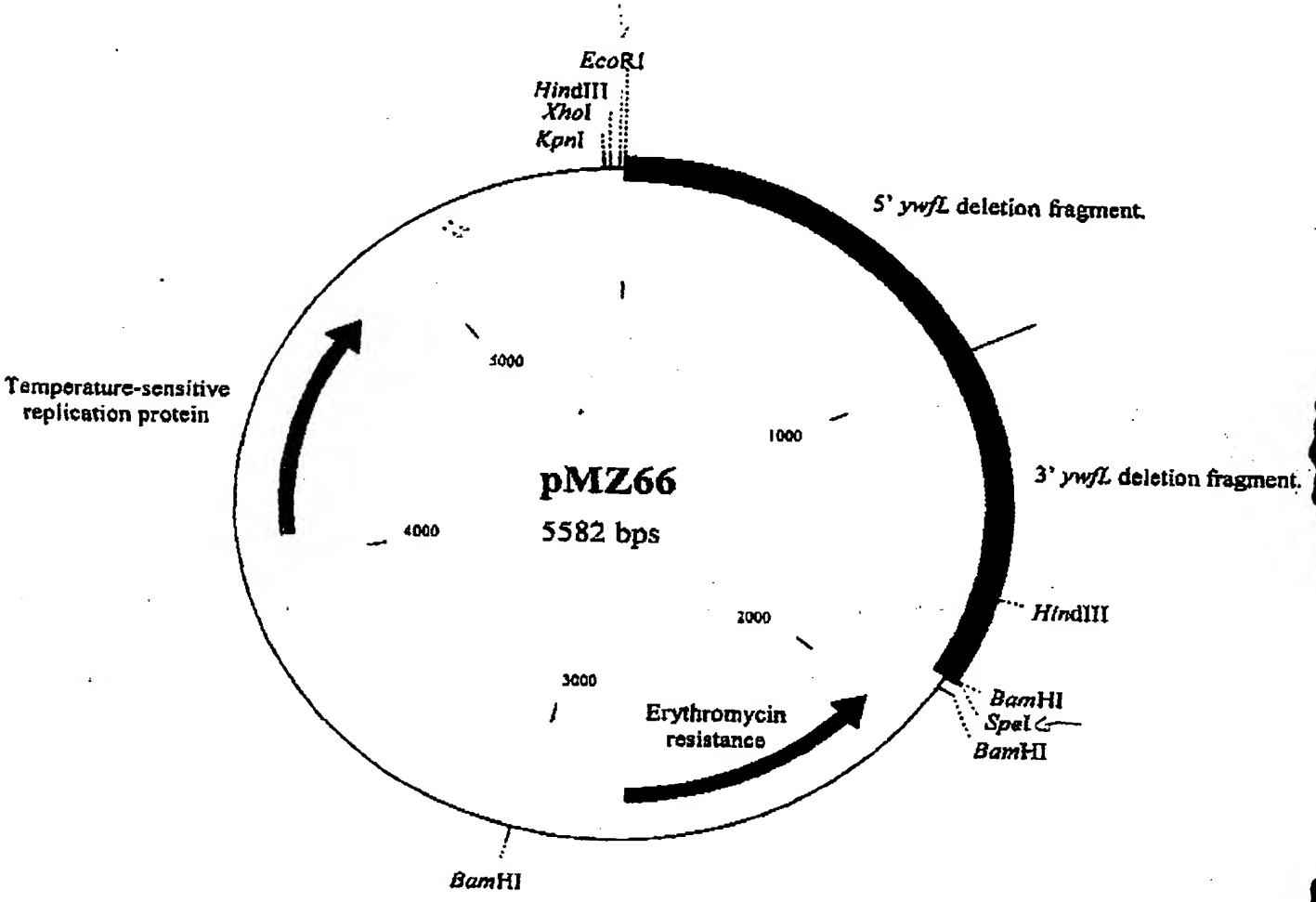
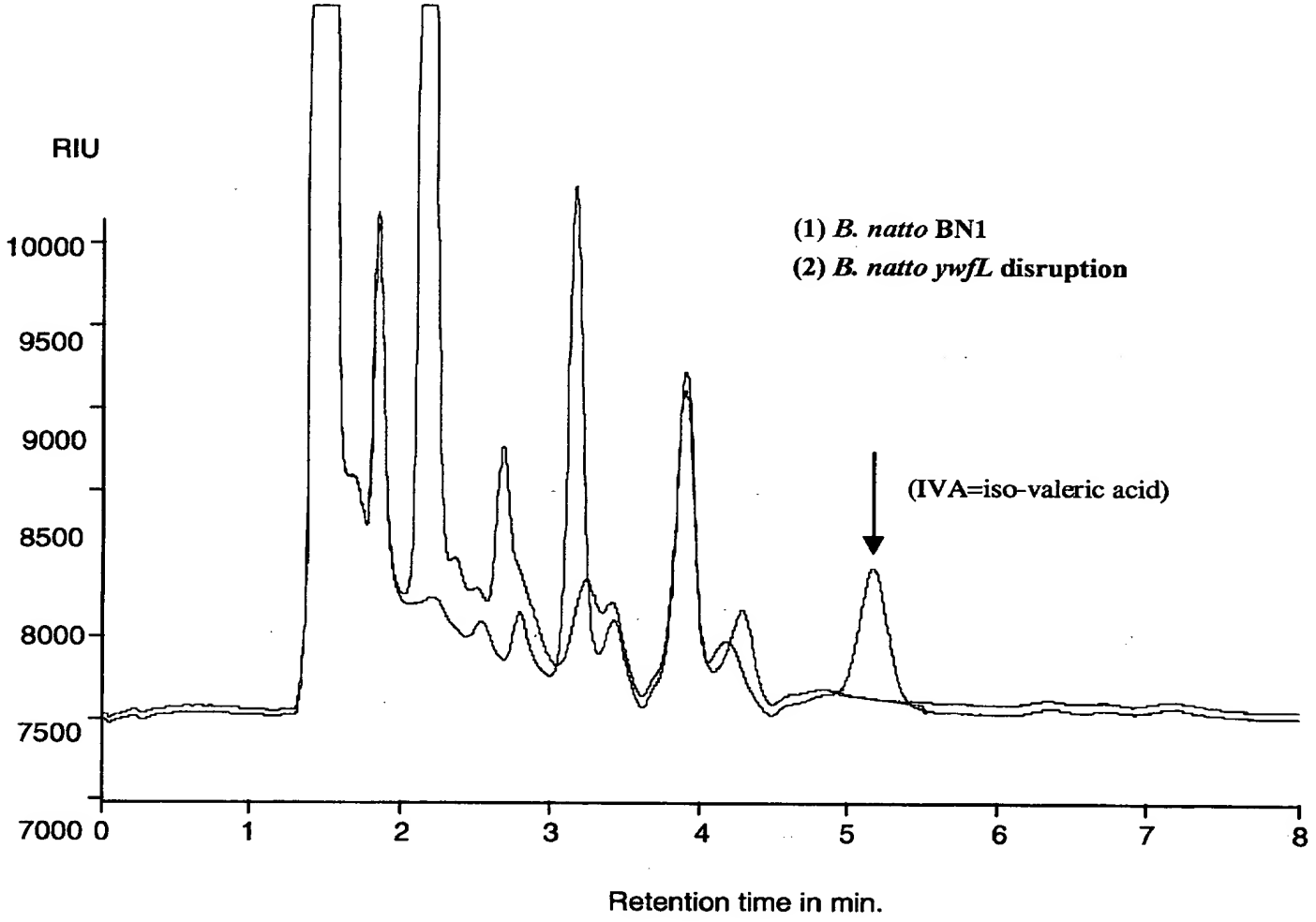


Figure 3 Chromatogram of fermentation products in the culture medium produced by the wild-type *B. natto* and the *ywfL* disruption iso-genic derivative.



Summary

The present invention pertains to novel strains of the Bacillus subtilis group capable of fermenting beans, which are essentially devoid of any iso-valeric acid production. The present invention especially relates to novel strains of Bacillus natto, in which one or more genes involved in the biosynthetic pathway for the production of iso-valeric acids are essentially non-functional.

Sequence Listings

(1) GENERAL INFORMATION:

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(ii) TITEL OF THE INVENTION : Novel strains of the Bacillus subtilis group for food fermentation

(iii) NUMBER OF SEQUENCES : 4

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(C) City: Munich
(D) State: Bavaria
(E) Coutry: Germany
(F) Regional Code: 81479

(v) COMPUTER READABLE FORM:
(A) Medium type: Floppy Disk
(B) Computer: IBM PC Compatibel
(C) System: PC-DOS/MS-DOS
(D) Software: Patentin Release Nr. 1, Version Nr. 1,25

(vi) INFORMATION OF THE APPLICATION:
(A) Application number: to be obtained
(B) Application date: herwewith
(C) Classification:

(vii) PRIORITY DATES: none

(viii) Attorney/Agent Informations
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(2) INFORMATION FOR SEQ ID. NO. 1:
(i) SEQUENCE CHARACTERISTICS:
(A) length: 30 nucleotides
(B) type: nucleic acid
(C) strandedness: single stranded
(D) topology: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQENCE DESCRIPTION: SEQ ID. NO.1:

GCGGCGGATC CGCTGATGAT CTCCCAGCCC 30

(3) INFORMATION FOR SEQ ID. NO. 2:
(i) SEQUENCE CHARACTERISTICS:
(A) length: 44 nucleotides
(B) type: nucleic acid
(C) strandedness: single stranded
(D) topology: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQENCE DESCRIPTION: SEQ ID. NO.2:

CTCAAATTCC ATTTCCTCAT CAGGACATGC ATAGCGTATC ATCC 44

(3) INFORMATION FOR SEQ ID. NO. 3:
(i) SEQUENCE CHARACTERISTICS:
(A) length: 31 nucleotides
(B) type: nucleic acid
(C) strandedness: single stranded
(D) Topology: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQENCE DESCRIPTION: SEQ ID. NO.3:

GGGGTCGAAT TCCACGAGAT ATCTAACTGC C 31

(4) INFORMATION FOR SEQ ID. NO. 4:
(i) SEQUENCE CHARACTERISTICS:
(A) length: 44 nucleotides
(B) type: nucleic acid
(C) strandedness: single stranded

(D) Topology: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQENCE DESCRIPTION: SEQ ID. NO.4:

GGATGATACG CTATGCATGT CCTGATGAGG AAATGGAATT TGAG 44